dehydrogenase and glucose 6-phosphate-dehydrogenase activity; both contain fumarase and aconitase, although aconitase activity is particularly low in the blood-stream form. Culture trypanosomes show pyruvic-oxidase activity, whereas blood-stream forms do not. Attempts to demonstrate transhydrogenases have been inconclusive.

I thank Mrs Winifred Jones and her colleagues for the preparation of the large quantities of medium involved.

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Studies on the Chemical Basis of the Antigenicity of Proteins

5. SYNTHESIS, CHARACTERIZATION AND IMMUNOGENICITY OF SOME MULTICHAIN AND LINEAR POLYPEPTIDES CONTAINING TYROSINE*

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The purpose of the present series of investigations is to determine the minimum definitive chemical features necessary to endow a molecule with immunogenicity, i.e. the capacity to elicit antibodies in an animal. It was also desired to elucidate the antigenic specificity relationships in the materials studied. Here we report results of immunological studies of some synthetic polypeptide antigens.

In discussing immunogenicity and antigenic specificity, one may draw a parallel with enzymes, in which a certain portion of the molecule may be assumed to possess catalytic activity, and another portion, sometimes even overlapping or coinciding with the first, may confer substrate specificity. For antigens, one might differentiate between a moiety of the molecule necessary for immunogenicity and another responsible for serological specificity.

Sela & Arnon (1960*a*, *b*) and Arnon & Sela (1960*b*) showed that gelatin modified by attachment to its amino groups of short peptide chains of tyrosine, tryptophan, phenylalanine and, to a less extent, cysteine, had considerably greater immunogenicity than unmodified gelatin had. The covalent binding of peptides containing alanine, lysine, glutamic acid, serine or proline did not increase the immunogenicity as compared with gelatin. The increase in the immunogenicity through attachment of the aromatic amino acids is not due to their aromatic character. This was demonstrated by showing that the attachment of fully saturated cyclohexylalanine (Sela & Arnon,

* Part 4: Arnon & Sela (1960*a*).

1960*c*) also resulted in a considerable increase in immunogenicity (Sela & Arnon, 1960*b*). It was suggested that enhancement of immunogenicity was due to an increase in rigidity of part of the active site in the molecule.

The new antigens possessed a sharp specificity, which was determined by the length of the peptide chains attached to gelatin. Thus a limited enrichment of gelatin with tyrosine resulted in an increase in immunogenicity without abolishing the serological specificity of gelatin. A more extensive tyrosination yielded antigens whose specificity resided exclusively in the newly attached peptide chains (Arnon & Sela, 1960*b*). Whereas the attachment of polar amino acid residues to gelatin caused almost no change in extent of immunogenicity, the enrichment of gelatin with both tyrosine and glutamic acid yielded a powerful antigen of very narrow specificity. This agrees with the common assumption that polar groups strongly influence the antigenic specificity of proteins.

In some of the above-mentioned cases the specificity was due entirely to the chemically introduced polypeptides. The question arose therefore whether in these cases gelatin still contributes towards the immunological properties of the new antigens, or whether it is simply an inert carrier. Such a carrier might be replaced by a synthetic material, thus yielding completely synthetic antigens. We have therefore prepared a multichain copolymer in which chains of polypeptides containing L-tyrosine and L-glutamic acid were built on multichain poly-DL-alanine (Sela, 1954; Sela, Katchalski & Gehatia, 1956). [The term 'multichain polymer' was introduced by Schaefgen & Flory (1948) to denote branched polymers whose molecules are composed of linear polymeric chains attached to a polyfunctional core.] The material obtained was shown to be a powerful, sharply specific antigen in rabbits (Sela & Arnon, 1960*d*, *e*). Gill & Doty (1960) reported the immunogenicity of a synthetic linear polypeptide containing tyrosine. Previous efforts to detect an immune specific response to homopolymers and heteropolymers of amino acids have been summarized by Sela & Katchalski (1959), Sela & Arnon (1960*a*) and Sela (1962).

The observation that a completely synthetic material composed of α-amino acids bound through peptide linkages can be a potent, specific antigen permits a systematic inquiry into the chemical basis of antigenicity. The synthetic approach offers the advantage that, once a biological property has been demonstrated unequivocally, a greater number of suitable analogues with limited variations in structure may be prepared and tested. In this way one may relate specific structural features to biological function.

The present paper is concerned mainly with the role of the overall shape and of the size of the molecule, as well as with the locus in the molecule of the amino acid important for immunogenicity. Brief preliminary reports have been published (Sela, Arnon & Fuchs, 1961; Sela, 1962). In the studies described below this critical amino acid was tyrosine. Although tyrosine is an adequate immunogenic factor, it is not implied that it is unique. Recent contributions to the subject of synthetic polypeptide antigens are: Gill & Doty (1961*a*, *b*, 1962); Gill & Dammin (1962); Maurer (1961, 1962).

This paper presents evidence that the immunogenically important area of the molecule must be accessible to the site of the biosynthesis of the antibody. The overall shape of the molecule does not seem to be an important factor in determining the immunogenicity, as both linear and multichain polymers may be immunogenic. It may also be concluded from the studies reported below that synthetic materials with molecular weights as small as 4000 may be good antigens.

MATERIALS

Linear polymers of α-amino acids were prepared from the monomeric units, the *N*-carboxy-α-amino acid anhydrides, and multichain polyamino acids were synthesized by the reaction of monomers with multifunctional linear polymers. To avoid a cumbersome nomenclature of the polymers, the following conventions were adopted in this paper: homopolymers are denoted by the letter p followed by the abbreviation of the amino acid composing it (thus pLys stands for polylysine); a random copolymer is described by the letter p followed by the abbreviations of the amino acids composing it, separated by commas and enclosed in parentheses [thus p(Lys,Ala) stands for a copolymer of Lys and Ala]; the link between the side-chain polymer and the backbone polymer is indicated by two hyphens; the link between the side-chain polymer and a polypeptide attached to extend it is denoted by one hyphen. According to this simplified nomenclature our first synthetic antigen is denoted: p(Tyr,Glu)-pAla--pLys. A schematic representation of some multichain copolymers of amino acids is given in Fig. 1.

The samples of gelatin, polyglutamyl gelatin (pGluGel), egg albumin, polytyrosyl egg albumin, linear and multichain poly-L-glutamic acids, as well as copolymers of L-tyrosine and L-glutamic acid in residue molar ratios of 1:1·1 (number-average degree of polymerization, *n*, 31) and 1:9 (*n* 88) were described by Sela & Arnon (1960*a*). The polytyrosyl gelatins (pTyrGel A, pTyrGel B, pTyrGel C) and copoly-(L-glutamyl, L-tyrosyl)gelatin [p(GluTyr)Gel] were described by Arnon & Sela (1960*b*).

Linear poly-α-amino acids

A series of linear polymers was prepared (Table 1) for study of immunogenicity and antigenic specificity or as backbones in the synthesis of multichain polyamino acids. The *N*-carboxyanhydrides of L-tyrosine (Berger *et al.* 1958),

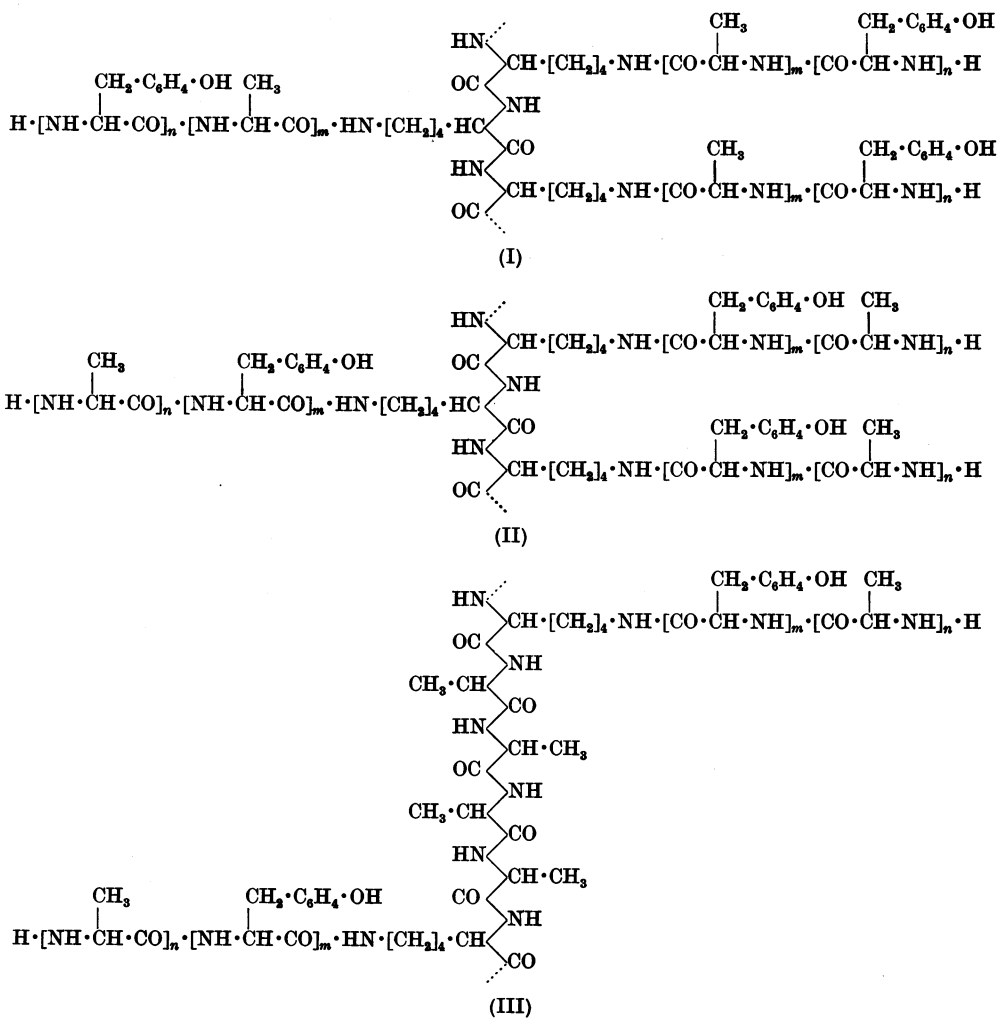

Fig. 1. A schematic representation of some multichain copolymers of amino acids. (I) pTyr-pAla--pLys. (II) pAla-pTyr--pLys. (III) pAla-pTyr--p(Lys,Ala); the molar residue ratio of lysine to alanine in the backbone of the polymer is 1:4.

Table 1. *Linear copolymers of α-amino acids*

No. and designation of sample	Copolymer of	Molar ratio of *N*-carboxy-amino acid anhydrides in the polymerization mixture	Molar ratio of amino acid residues in the copolymer	Wt. (%) of tyrosine residues in the copolymer	Number-average degree of polymerization*
2, p(Lys,Ala)	L-Lys, DL-Ala	1:0·8	1:1	—	60
3, p(Lys,Ala)	L-Lys, DL-Ala	1:4	1:3·9	—	70
4, p(Lys,Ala)	L-Lys, DL-Ala	1:8	1:6	—	45
42, p(Tyr,Glu,Ala)†	L-Tyr, L-Glu, L-Ala	1:5:4	1:5·4:3·9	15	40
44, p(Tyr,Glu,Ala)†	L-Tyr, L-Glu, L-Ala	1:1:1·3	1:1·2:1·1	41	145
102, p(Tyr,Glu)†	L-Tyr, L-Glu	1:1	1:1	56	85
103, p(Tyr,Glu,Ala)†	L-Tyr, L-Glu, DL-Ala	1:1·3:1	1:1·4:0·8	41	55

* From amino-nitrogen determinations. For copolymers containing lysine, the analysis was carried out before the removal of the benzyloxycarbonyl group from the ε-amino function of lysine.

† Tested for immunogenicity.

γ-benzyl-L-glutamate (Katchalski & Berger, 1957), DL-alanine (Sela & Berger, 1955), L-alanine (Bailey, 1950) and ε-*N*-benzyloxycarbonyl-L-lysine (Bergmann, Zervas & Ross, 1935; Katchalski & Sela, 1958) were used as monomers. The last-named anhydride was obtained from ε-*N*-benzyloxycarbonyl-L-lysine by the usual Fuchs–Farthing reaction in phosgene. ε-*N*-Benzyloxycarbonyl-L-lysine was prepared by coupling an equimolar amount of sodium salt of L-lysine with benzyl chloroformate in cold chloroform solution (Katchalski & Sela, 1958). All the *N*-carboxy-α-amino acid anhydrides were recrystallized repeatedly until the chlorine content was less than 0·03%. Poly-L-lysine hydrobromide samples were prepared by a modification of the procedure of Katchalski (1957), *n*-butylamine being used as the initiator of polymerization.

The synthesis of copolymers was carried out at room temperature in anhydrous dioxan (1 g. of *N*-carboxy-α-amino acid anhydride/20 ml. of dioxan), triethylamine being used as initiator, except for the copolymers of L-lysine and DL-alanine, where the polymerization reaction was initiated with diethylamine. The amounts of the *N*-carboxyamino acid anhydrides used in the various preparations are given in Table 1. Four days after the start of the reaction, the polymers were precipitated with water and dried *in vacuo* over phosphorus pentoxide. The benzyloxycarbonyl groups were removed from the copolymers with anhydrous hydrogen bromide in acetic acid for 30 min. at room temperature (Ben-Ishai & Berger, 1952). The unprotected copolymer hydrobromides were precipitated and purified similarly to polylysine hydrobromide (Katchalski, 1957). The benzyl groups were removed from the copolymers by treatment with anhydrous hydrogen bromide in acetic acid for 72 hr. at 2° (Yaron & Berger, 1958). The precipitation of the copolymers was completed with anhydrous ether; the copolymers obtained were washed with anhydrous ether and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. All the copolymers tested for their immunogenicity were dialysed against three changes of distilled water at 2° for 24 hr. The contents of the dialysis bags were freeze-dried and stored at 2°.

For analysis, the copolymers of lysine and alanine were hydrolysed with 6N-hydrochloric acid for 24 hr., and the other copolymers were hydrolysed with concentrated hydrochloric acid for 48 hr. The hydrolysis was carried out in sealed evacuated tubes at 105°. The amino acid contents of the various copolymers were determined by ninhydrin colorimetry (Kay, Harris & Entenman, 1956) of the hydrolysates after chromatography in butan-1-ol–acetic acid–water (50:12:50, by vol.). The content of tyrosine was also calculated from the spectral absorption at 293·5 mμ in a solution of pH 13; it checked well in each case with the result obtained by chromatographic analysis. The number-average degrees of polymerization were obtained from amino nitrogen (Van Slyke) determinations.

Multichain polyamino acids

A series of multichain polymers was prepared (Table 2), multifunctional branched polymers being used as initiators of polymerization. Multipoly-DL-alanyl-poly-L-lysine (multichain poly-DL-alanine; pAla--pLys) was prepared from *N*-carboxy-DL-alanine anhydride and poly-L-lysine as previously reported (Sela *et al.* 1956). The multifunctional amine initiator [e.g. poly-L-lysine (pLys) or multichain poly-DL-alanine (pAla--pLys)] was dissolved in 0·05M-phosphate buffer, pH 7·0, and treated at 2° with the desired *N*-carboxy-α-amino acid anhydrides in dioxan (1 g. of *N*-carboxyamino acid anhydride/20 ml. of dioxan) (see Table 2). After 48 hr. at 2° the reaction product was dialysed against distilled water for 3 days. The contents of the dialysis bag were freeze-dried and stored at 2°. When γ-benzyl-*N*-carboxy-L-glutamate anhydride was present in the polymerization mixture, the reaction product precipitated out of solution. The precipitation was completed in these cases with cold acetone. The precipitates were dried over sulphuric acid and phosphorus pentoxide. The benzyl groups were removed, and the debenzylated multichain copolymers were purified and dried as described above for the linear copolymers. The multichain polypeptides obtained were dissolved in aqueous 0·05N-sodium hydrogen carbonate, dialysed against distilled water for 3 days, freeze-dried and stored at 2°.

The multichain copolymers were hydrolysed, and their amino acid contents determined as described above for the linear copolymers. In experiments concerned with the role of the locus of tyrosine within the molecule in conferring immunogenicity, it was of considerable importance to elucidate whether the amino-terminal groups of the polymeric side chains of the alanylated branched polymers (samples nos. 16, 18, 27, 28, 30; see Table 2) were solely those of alanine. For this purpose all the copolymers tested for immunogenicity, possessing polyalanine on the outside of their side chains, were treated with dinitrofluorobenzene by the method of Levy (1954) and Anfinsen, Sela & Tritch (1956). The resulting dinitrophenyl polymers, after hydrolysis, were analysed for amino-terminal groups by two-dimensional paper chromatography (Pairent & Williamson, 1960). Only *N*-(dinitrophenyl)alanine was detected in all the copolymers tested.

Physicochemical characterization

Under the conditions used in the preparation of the polymers described, the termination reaction (Sela & Berger, 1955) occurs only to a negligible extent (Katchalski & Sela, 1958). In its absence, it was predicted from a theoretical analysis of the kinetics of polymerization of linear and multichain polyamino acids that both the linear and the multichain polymers obtained should possess a relatively narrow distribution of molecular weight (Katchalski, Shalitin & Gehatia, 1955; Katchalski, Gehatia & Sela, 1955). This was demonstrated through physicochemical studies of linear (Katchalski & Sela, 1958; Daniel & Katchalski, 1962) and multichain (Sela *et al.* 1956; Berger & Yaron, 1962) polymers as well as recent chromatographic and ultracentrifugal studies of a polypeptidyl protein, the polyalanyl ribonuclease (Anfinsen, Sela & Cooke, 1962).

Several of the polymers described above were subjected to sedimentation and diffusion in the ultracentrifuge. The experiments were performed on 0·5% solutions of the polymers in 0·1M-phosphate buffer, pH 7·0 (obtained by mixing 39·0 ml. of 0·1M-monobasic sodium phosphate and 61·0 ml. of 0·1M-dibasic sodium phosphate). A typical sedimentation pattern is shown in Fig. 2. The sedimentation and diffusion coefficients are given in Table 3. For calculation of the average molecular weights the partial specific volumes of the copolymers are computed from the partial specific volumes of the amino acid residues and their proportion by weight in the polymer. The partial specific

Table 2. *Preparation and amino acid content of multichain copolymers*

No. and designation of sample	Multifunctional initiator: No. and designation	Multifunctional initiator: Wt. (g.) and vol. (ml.) of buffer	Amount (g.) of *N*-carboxyanhydrides of: γ-Benzyl-L-Glu	Amount (g.) of *N*-carboxyanhydrides of: DL-Ala	Molar ratio of amino acid residues in the copolymer: L-Lys	L-Tyr	L-Glu	DL-Ala	Wt. (%) of tyrosine residues in the copolymer
5, pAla--pLys*	pLys	2; 500	—	40	1	—	—	24	—
20, pAla--pLys†	pLys	0·25; 100	—	6	1	—	—	17·6	—
19, p(Tyr,Glu)-pAla--pLys†	20, pAla--pLys	1·2; 60	0·62	—	1	1·1	2·2	17·5	9·9
35, p(Tyr,Glu)-pAla--pLys*†	5, pAla--pLys	1; 80	0·8	—	1	1·8	3·7	24	11·3
33, pTyr-pAla--pLys*†	5, pAla--pLys	2; 150	—	—	1	1·7	—	24·7	12·9
34, pGlu-pAla--pLys*†	5, pAla--pLys	2; 150	1·6	—	1	—	2·9	25	—
21, pTyr--pLys*	pLys	1; 35	—	—	1	0·9	—	—	53·5
27, pAla-pTyr--pLys*†	21, pTyr--pLys	1·1; 80	—	8	1	1	—	23	8·5
31, pGlu-pAla-pTyr--pLys*†	27, pAla-pTyr--pLys	1·5; 70	0·6	—	1	0·93	2·26	20	7·4
22, p(Tyr,Glu)--pLys*†	pLys	2; 100	5	—	1	1·3	2·2	—	34
28, pAla-p(Tyr,Glu)--pLys*†	22, p(Tyr,Glu)--pLys	0·7; 50	—	3·5	1	1·2	2·0	15·7	11·5
106, p(Tyr,Ala)--pLys*†	pLys	2; 140	2·3	—	1	1·3	—	1·3	49
11, pTyr--p(Lys,Ala)	3, p(Lys,Ala) (1:3·9)‡	0·2; 40	—	—	1	2·3	—	3·7	49
16, pAla-pTyr--p(Lys,Ala)†	11, pTyr--p(Lys,Ala)	0·15; 60	—	1·5	1	2·5	—	22·4	19·2
117, p(Tyr,Glu)--p(Lys,Ala)	3, p(Lys,Ala) (1:3·9)‡	2; 120	2·5	—	1	1·35	2·1	4	24·4
120, pAla-p(Tyr,Glu)--p(Lys,Ala)†	117, p(Tyr,Glu)--p(Lys,Ala)	1; 70	—	6	1	1·65	2·0	39	7·9
13, pTyr--p(Lys,Ala)	4, p(Lys,Ala) (1:6)‡	0·2; 40	—	—	1	3·4	—	6	50
18, pAla-pTyr--p(Lys,Ala)†	13, pTyr--p(Lys,Ala)	0·15; 60	—	1·5	1	3·6	—	30·2	20·5
8, pAla--p(Lys,Ala)	4, p(Lys,Ala) (1:6)‡	0·4; 60	—	3	1	—	—	34	—
26, p(Tyr,Glu)-pAla--p(Lys,Ala)†	8, pAla--p(Lys,Ala)	1; 100	0·5	—	1	1·7	2·2	30	9·9
23, p(Tyr,Glu)--p(Lys,Ala)†	4, p(Lys,Ala) (1:6)‡	3; 100	2·5	—	1	1·3	1·9	5·5	22
30, pAla-p(Tyr,Glu)--p(Lys,Ala)†	23, p(Tyr,Glu)--p(Lys,Ala)	0·7; 50	—	3	1	1·4	2·1	25	9·5

* Derived from the same batch of poly-L-lysine hydrobromide.
† Tested for immunogenicity.
‡ Residue molar ratio: these backbone copolymers are described in Table 1.

volume of the alanine residue was taken as 0·72 (as found for multichain polyalanine by Sela *et al.* 1956), that of tyrosine residue as 0·71 (Cohn & Edsall, 1943), that of lysine residue as 0·72 (as found for polylysine hydrochloride by Applequist, 1959) and that of sodium glutamate residue as 0·57 (as suggested by Friedman, Gill & Doty, 1961). As the values given for sedimentation and diffusion coefficients are not intrinsic, the molecular weights calculated from them should be considered only approximate, and the true values might be somewhat higher. In the few cases where intrinsic sedimentation coefficients were obtained, the difference in molecular weights computed from intrinsic and 0·5% concentration values was less than 15%.

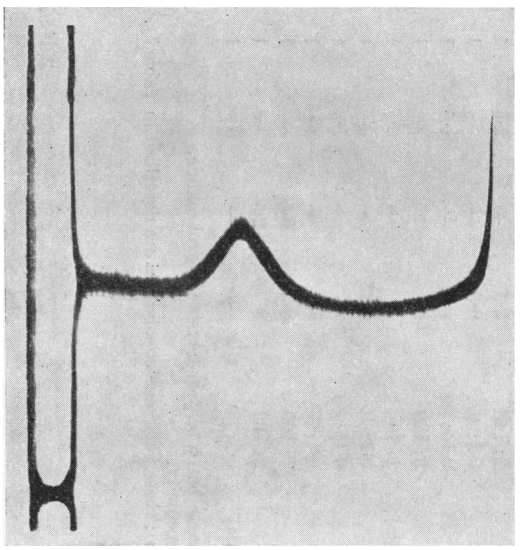

Fig. 2. A sedimentation pattern, obtained in a Spinco model E ultracentrifuge, of sample 19, p(Tyr,Glu)-pAla--pLys (0·5%, w/v, in 0·1 M-phosphate buffer, pH 7·0). The photograph was taken 96 min. after full speed (56 100 rev./min.) was attained. Centrifugal movement is from left to right.

Labelling of antigens

Samples 42, p(Tyr,Glu,Ala), 44, p(Tyr,Glu,Ala), 102, p(Tyr,Glu), 19, p(Tyr,Glu)-pAla--pLys, 22, p(Tyr,Glu)--pLys, and 30, pAla-p(Tyr,Glu)--p(Lys,Ala), were iodinated with ^{131}I (50 μc/10 mg.) according to Talmage, Baker & Akeson (1954). The substances, isolated after exhaustive dialysis, had radioactivities in the range 500 000–1 000 000 counts/min./mg.

METHODS

Immunization procedure. The antigens used were incorporated in a water-in-oil adjuvant mixture, according to Salk & Laurent (1952). Equal parts of 2·5% antigen solution in aqueous 0·9% sodium chloride and ready-prepared complete Freund adjuvant from Difco Laboratories, Detroit, Mich., U.S.A., were homogenized by repeated filling and forcible ejection from a syringe.

Each material tested was injected into four rabbits. After pre-immunization bleedings, the antigen was administered into the thighs of the hind legs of the animals. Three injections of 1·0 ml. of the adjuvant mixture were given at fortnightly intervals. If no positive response was obtained at this stage, one additional injection of adjuvant mixture was administered. If a negative response still persisted, the animal was injected intravenously for 4 consecutive days with 0·5 ml. of 0·2% antigen solution in aqueous 0·9% sodium chloride.

The animals were bled weekly, starting 2 weeks after the third injection. Thiomersal was added to all sera to a concentration of 0·01%.

Quantitative precipitin studies. In preliminary tests qualitative precipitin reactions were carried out on sera of individual rabbits. When the immune response was positive, at least two of the four animals gave homologous precipitin reactions. Thereafter, such antisera were pooled before quantitative studies. Several pools of the antisera to the same antigen were collected at various times.

In all cases, increasing amounts of antigen dissolved in equal volumes of aqueous 0·9% sodium chloride were added to test tubes containing a constant volume of serum (0·3, 0·5 or 1·0 ml., depending on the immune response). The final volume never exceeded 1·5 ml. In control experiments aqueous 0·9% sodium chloride solution was added in the absence of antigen. The contents of the tubes were mixed, placed in a water bath at 37° for 1 hr., and then in

Table 3. *Physicochemical properties of some copolymers*

No. and designation of sample	Partial specific vol. ($\bar{v}$)	$10^{13}\times$ Sedimentation coefficient ($S_{20,w}$)	$10^{7}\times$ Diffusion coefficient ($D_{20,w}$)	Average mol.wt.*
42, p(Tyr,Glu,Ala)	0·63	0·90	14·40	4 100
5, pAla--pLys	0·72	2·26	6·20	31 600
19, p(Tyr,Glu)-pAla--pLys	0·70	2·97	3·43	70 000
35, p(Tyr,Glu)-pAla--pLys	0·69	2·96	6·65	35 000
33, pTyr-pAla--pLys	0·72	3·30	7·20	39 800
34, pGlu-pAla--pLys	0·695	2·89	5·30	43 500
27, pAla-pTyr--pLys	0·72	3·00	8·20	31 600
22, p(Tyr,Glu)--pLys	0·65	2·00	13·60	10 200
23, p(Tyr,Glu)--p(Lys,Ala)	0·68	0·95	7·15	10 100

* Calculated from sedimentation and diffusion coefficients.

the cold room for 24 hr. or, in some cases, for 48 hr. The tubes were centrifuged and precipitates which formed were washed three times with chilled (2°) aqueous 0·9% sodium chloride, and dissolved in 0·1 N-sodium hydroxide (1·1 ml.). The extinction of these solutions was read at 2800 Å. The precipitin reaction of sample 102, p(Tyr,Glu), was followed by readings of E at 2800 Å of solutions of the precipitates both in aqueous 0·25 N-acetic acid and in 0·1 N-sodium hydroxide. No significant difference was found between amounts of antibody calculated from either measurement.

The amount of antigen in the precipitates was determined in several cases after labelling with ^{131}I. The antibody content was then obtained from the measured E after deducting the calculated E of the antigen.

Radioactivity. Radioactivity of antigen–antibody precipitates, as well as of the supernatant fluids, was measured in a well-type Tracerlab Scintillation Counter.

Immunodiffusion. Some antigens and their antisera were subjected to double diffusion in agar gel, either on plates (Ouchterlony, 1953) or in tubes, making use of a modification (Oakley & Fulthorpe, 1953) of the Oudin (1952) technique. The double diffusion on cellulose acetate strips (Kohn, 1960) was also used.

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approximately 25°, with quartz cells of 1 cm. light-path. In the readings of solutions of antigen–antibody precipitates, cells with a capacity of 1 ml. were used.

Sedimentation analysis. This was carried out in a Spinco model E ultracentrifuge, at 20–22° with the schlieren optical system. The samples were sedimented at 56 100 rev./min. The results were corrected to 20°. Sample 42, p(Tyr,Glu,Ala), was sedimented in a synthetic-boundary cell (Pickels, Harrington & Schachman, 1952) at 59 780 rev./min.

Diffusion measurements. These were performed in the same Spinco model E ultracentrifuge (Daniel & Katchalski, 1962). The boundary between the solvent and the solution was obtained with a synthetic-boundary cell and operating at low gear (9341 rev./min.). At this speed the sedimentation of the polymers investigated was practically negligible.

RESULTS

The polymers tested for immunogenicity in rabbits are designated in Tables 1 and 2. None of the materials gave any precipitate when added to pre-immunization sera, except for two multichain polymers containing tyrosine but no glutamic acid (molecules with overall positive charge at neutral pH values). These polymers [33, pTyr-pAla--pLys; 106, p(Tyr,Ala)--pLys] caused some precipitation when added in amounts higher than 0·2 mg./ml. of normal serum. Sera from immunized animals were subjected to the precipitin reaction. All sera that gave a negative reaction with homologous substance were checked for possible presence of antibodies by means of cross-reactions with proteins such as egg albumin and gelatin, peptidylated proteins [pTyrGel, pGluGel, p(GluTyr)Gel, polytyrosyl egg albumin] as well as with various linear and multichain copolymers mentioned in Tables 1 and 2. No cross-precipitation was observed with any of the sera that gave a negative homologous reaction.

Immunogenicity of multichain polypeptides containing tyrosine on the outside of the molecule

In our search for an adequate 'inert carrier' we chose multichain poly-DL-alanine. In contrast with polymers of L-alanine, which are insoluble in water, polymers of DL-alanine are water-soluble (Katchalski & Sela, 1958). No antibodies to multichain poly-DL-alanine (20, pAla--pLys) were detected when the sera were tested either by the usual precipitin reaction or by Maurer's (1954) modification. When the poly-DL-alanine side chains in the multichain polymer were extended with peptides containing L-tyrosine and L-glutamic acid, the resulting copolymers, p(Tyr,Glu)-pAla--pLys, samples 19 and 35 (Tables 2 and 3), exhibited a characteristic immunogenic behaviour; this is illustrated with sample 19 in Table 4 and Fig. 3. To determine directly the antigen in the specific precipitate, sample 19 was trace-iodinated with ^{131}I before precipitation. The system 35–anti-35 showed a maximum in the precipitin curve at 50 μg. of antigen/ml. of antiserum. In this case the precipitate contained 395 μg. of antibody.

Several polymers of the general formula p(Tyr, Glu)-pAla--p(Lys,Ala) also elicited a positive antigenic response. These polymers are similar in structure to p(Tyr,Glu)-pAla--pLys, but the side chains are attached to a backbone composed of linear copolymers of L-lysine and DL-alanine in various residue molar ratios. Thus the average distance between the side chains is increased in these branched polymers in comparison with p(Tyr,Glu)-pAla--pLys. The precipitin curve of 26, p(Tyr,Glu)-pAla--p(Lys,Ala), is given in Fig. 4.

To elucidate whether both tyrosine and glutamic acid are necessary for conversion of a non-immunogenic branched polymer into an immunogen, substances in which either tyrosine or glutamic acid peptides were attached to the multichain poly-alanine [33, pTyr-pAla--pLys (see Fig. 1); 34, pGlu-pAla--pLys] were studied for immunogenic capacity. Sample 33 gave rise to formation of antibodies (Fig. 4), demonstrating that tyrosine by itself has the capacity to endow a macromolecule with immunogenicity. Sera of animals injected with 34, pGlu-pAla--pLys, gave a negative response when examined either with homologous substance or with linear poly-L-glutamic acid, multichain poly-L-glutamic acid, poly-L-glutamyl gelatin as well as the proteins gelatin, edestin and egg albumin.

A positive immunological response was obtained after injecting 22, p(Tyr,Glu)--pLys, a substance of relatively low molecular weight (Table 3), in

Table 4. *Composition of precipitates of the systems* 19 [*p(Tyr,Glu)-pAla--pLys–anti-p(Tyr, Glu)-pAla--pLys*] *and* 22 [*p(Tyr,Glu)--pLys–anti-p(Tyr,Glu)--pLys*]

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated* (μg.)	Antigen precipitated (%)	Antibody precipitated†	Antibody/ antigen in precipitate (w/w)
19, p(Tyr,Glu)-pAla--pLys	12·5	12·35	99·0	195	15·4
	31·2	26·5	85·0	321	12·1
	62·5	47·2	75·5	418	8·9
	125	70·0	56·0	468	6·7
	250	80·8	32·3	412	5·1
	437	79·3	18·2	345	4·3
	750	77·5	10·4	260	3·4
	1250	70·3	5·6	212	3·0
22, p(Tyr,Glu)--pLys	2	1·42	71	84	59·2
	5	3·98	79·5	174	43·7
	10	7·61	76·1	285	37·5
	30	16·8	56	439	26·2
	50	18·85	37·7	451	23·9
	75	18·1	24·2	380	21
	100	18·85	18·9	382	20·2
	200	16·9	8·5	284	16·8
	300	16·4	5·5	216	13·2

* From radioactivity data.

† From *E* at 2800 Å of solutions of the precipitates in 0·1 N-sodium hydroxide after deducting the calculated *E* of the antigen.

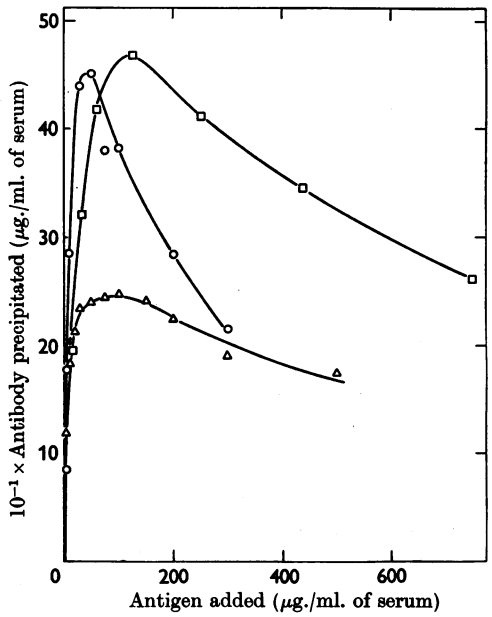

Fig. 3. Homologous precipitin curves of 19, p(Tyr,Glu)-pAla--pLys (□), 22, p(Tyr,Glu)--pLys (○), and 30, pAla-p(Tyr,Glu)--p(Lys,Ala) (△). The amount of antibody was obtained from *E* at 2800 Å after deducting the calculated *E* of the antigen. The amount of antigen in the precipitate was obtained from radioactivity data.

which peptides of tyrosine and glutamic acid are attached directly to polylysine (Table 4 and Fig. 3). Similarly, 23, p(Tyr,Glu)--p(Lys,Ala), is immunogenic, as shown in Fig. 4. The immunogenicity of a multichain polypeptide containing both L-tyrosine and DL-alanine in its side chains [106, p(Tyr, Ala)--pLys] is also illustrated in Fig. 4. Immunodiffusion of the p(Tyr,Glu)--pLys–anti-p(Tyr, Glu)--pLys system resulted in one line of precipitation on cellulose acetate strips as well as in agar-gel tubes or plates.

Immunogenic capacity of multichain polypeptides containing tyrosine on the inside of the molecule

The chemical structure of multichain polypeptides permits the evaluation of the role of the locus of the immunogenically important area (in our case tyrosine-containing peptides) within the molecule. Molecules may be synthesized similar in size, shape and amino acid composition to the antigens p(Tyr,Glu)-pAla--pLys and pTyr-pAla--pLys (Fig. 1) but with the tyrosine-containing peptides attached directly to polylysine and covered with polyalanine chains. No immune response was detected in the sera of animals injected either with 28, pAla-p(Tyr,Glu)--pLys, or with 27, pAla-pTyr--pLys (Fig. 1). Sample 31, pGlu-pAla-pTyr--pLys, was also non-immunogenic.

To determine whether the immunogenicity of the substances containing tyrosine on the outside of the molecule is due to the fact that they are at the amino terminus of the side chains, or whether it is due, in a more general way, to their accessibility to the biosynthetic site, the following series of experiments was performed. Multichain polymers were prepared in which the tyrosine peptides were still covered by polyalanine, but the entire side chains were attached to backbones composed of

copolymers containing L-lysine and DL-alanine in various residue molar ratios. Accordingly, the average distance between the side chains was increased considerably, presumably permitting a greater flexibility of the polymeric molecule. When the alanylated tyrosine–glutamic acid peptide side chains were bound to a polymer of lysine and alanine in a residue molar ratio of 1:6, the resulting 30, pAla-p(Tyr,Glu)--p(Lys,Ala), was shown to be immunogenic (Table 5 and Fig. 3), a finding in contrast with that for 28, pAla-p(Tyr,Glu)--pLys. Similarly, the attachment of alanylated tyrosine peptide side chains to a polymer of lysine and alanine in a residue molar ratio of 1:6 yielded an antigen, 18, pAla-pTyr--p(Lys,Ala) (Figs. 1 and 5), in contrast with 27, pAla-pTyr--pLys. Substances built in analogy to samples 30 and 18, but on a backbone composed of a polymer of lysine and alanine in a residue molar ratio of 1:3·9 (Table 2), namely 120, pAla-p(Tyr,Glu)--p(Lys,Ala), and 16, pAla-pTyr--p(Lys,Ala), were shown similarly to be immunogenic (Fig. 5). It should be stressed that the sera obtained after injection of samples 28 and 27 were not only unreactive towards the homologous substances, but also did not cross-react with antigens 30, 18, 120 and 16.

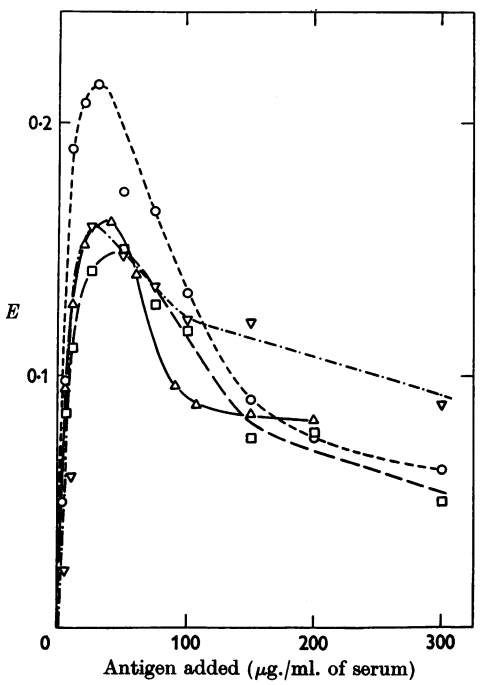

Fig. 4. Extinction at 2800 Å of solutions of homologous precipitates in 0·1 N-sodium hydroxide; ○, 23, p(Tyr,Glu)--p(Lys,Ala); □, 26, p(Tyr,Glu)-pAla--p(Lys,Ala); △, 33, pTyr-pAla--pLys; ▽, 106, p(Tyr,Ala)--pLys. Curves for the last two substances were corrected for some non-specific precipitation with normal serum.

Linear synthetic polypeptide antigens

In connexion with the immunological study of our first synthetic antigen, 19, p(Tyr,Glu)-pAla--pLys, we investigated the immunogenic behaviour of a copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio of 1:1·1 (*n* 31) (Sela & Arnon, 1960*c*). The substance gave a negative response when the sera were tested either by the usual precipitin reaction or by Maurer's modification (Maurer, 1954). No cross-reaction was obtained when these sera were checked with a variety of multichain copolymers containing tyrosine and glutamic acid on the outside of the polymeric molecule. On the other hand, a different batch of a copolymer of L-tyrosine and L-glutamic acid, of a higher degree of polymerization

Table 5. *Composition of precipitates of the system* 30 [*pAla-p(Tyr,Glu)--p(Lys,Ala)–anti-pAla-p(Tyr,Glu)--p(Lys,Ala)*]

Antigen added (μg./ml. of serum)	Antigen precipitated* (μg.)	Antigen precipitated (%)	Antibody precipitated† (μg.)	Antibody/antigen in precipitate (w/w)
5	4·35	87	118	27·1
10	7·17	71·7	183	25·6
20	11·2	56	213	19
30	16	53·5	234	14·6
50	20·8	41·6	240	11·5
75	27·6	36·8	244	8·85
100	31·4	31·4	247	7·85
150	35	28	241	6·9
200	42	21	225	5·35
250	44·5	17·8	216	4·85
300	45·2	15·1	190	4·2
500	42·6	8·5	175	4·1

* From radioactivity data.

† From *E* at 2800 Å of solutions of the precipitates in 0·1 N-sodium hydroxide after deducting the calculated *E* of the antigen.

Table 6. *Composition of precipitates of the systems* 102 [*p(Tyr,Glu)–anti-p(Tyr,Glu)*] *and* 42 [*p(Tyr,Glu, Ala)–anti-p(Tyr,Glu,Ala)*]

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated* (μg.)	Antigen precipitated (%)	Antibody precipitated† (μg.)	Antibody/ antigen in precipitate (w/w)
102, p(Tyr,Glu)	17	15·5	93·3	560	36
	33	30·7	92·2	1006	33
	67	52·8	79·2	1510	28·6
	100	75	75	1803	24
	133	86·5	64·8	2000	23·1
	167	94	56·3	2006	21·4
	200	102	51	1953	19·2
	233	106	45·5	1940	18·5
	267	108	40·5	1830	17
	333	106	31·8	1773	16·9
	417	110·5	26·6	1740	15·7
	500	108	21·6	1593	14·8
	666	85·6	12·8	1386	16·2
	1000	76·5	7·6	1050	13·7
	1666	63	3·8	690	11·3
	2666	55·5	2·1	446	8·1
42, p(Tyr,Glu,Ala)	5	2·8	56	273	97
	10	4·7	47	350	75·3
	20	7·2	36	422	44·3
	30	8·1	27	385	47·7
	50	8·8	17·6	403	46
	60	9·1	15·2	400	43·8
	80	8·6	10·7	346	40·4
	100	7·9	7·9	304	38·5
	150	7·1	4·7	265	37·3
	200	6·0	3	180	29·8
	300	4·8	1·6	155	32·4

* From radioactivity data.

† From E at 2800 Å of solutions of the precipitates in 0·1 N-sodium hydroxide after deducting the calculated E of the antigen.

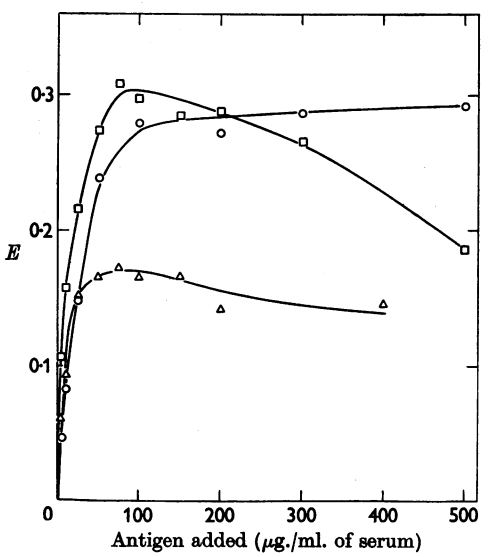

Fig. 5. Extinction at 2800 Å of solutions of homologous precipitates in 0·1 N-sodium hydroxide: □, 120, pAla-p(Tyr,Glu)--p(Lys,Ala); ○, 18, pAla-pTyr--p(Lys,Ala); △, 16, pAla-pTyr--p(Lys,Ala).

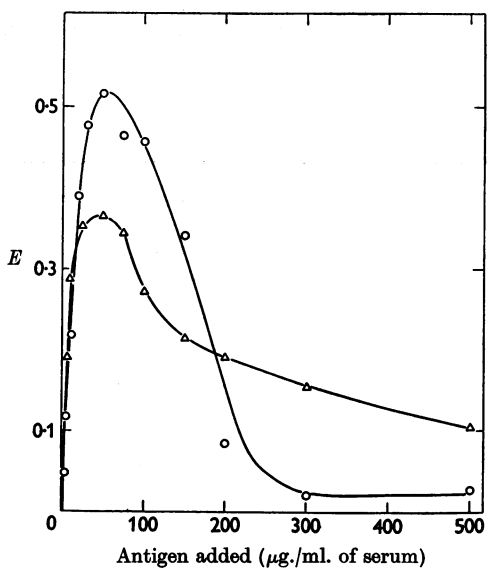

Fig. 6. Extinction at 2800 Å of solutions of homologous precipitates in 0·1 N-sodium hydroxide: ○, 44, p(Tyr,Glu, Ala); △, 103, p(Tyr,Glu,Ala).

[102, p(Tyr,Glu); 1:1; *n* 85] was shown to be immunogenic, as illustrated in Table 6.

Two copolymers of the three amino acids L-tyrosine, L-glutamic acid and L-alanine were tested for immunogenicity in rabbits and found to be antigenic. The homologous precipitin analysis for 42, p(Tyr,Glu,Ala), a polymer with a tyrosine weight content of 15 % and a molecular weight of approximately 4000, is given in Table 6, and the precipitin curve for 44, p(Tyr,Glu,Ala), is given in Fig. 6. The immunogenicity of a copolymer containing L-tyrosine, L-glutamic acid and DL-alanine [103, p(Tyr,Glu,Ala)] is also illustrated in Fig. 6.

DISCUSSION

The experiments on immunogenicity of synthetic polypeptides tested are summarized in Table 7, which includes both immunogenic and non-immunogenic substances. The results show that 15 of the synthetic polypeptides investigated are immunogenic, eliciting between 100 and 2000 μg. of precipitable antibody/ml. of serum. In none of the cases tested was all the antigen precipitated in the equivalence zone, the portion precipitable ranging from 65 % for the 102, p(Tyr,Glu), system to as little as 31 % for the 30, pAla-p(Tyr, Glu)--p(Lys,Ala), system. As no further precipitate occurs in this region on addition of either antigen or antibody to the supernatant fluid, it may be inferred, as with the results reported in connexion with studies on polypeptidyl gelatins (Sela & Arnon, 1960*a*; Arnon & Sela, 1960*b*) that the supernatant fluid contains soluble antigen–antibody complexes.

In Table 8 are given some calculated data on the weight and molar ratios of antibody to antigen for antigens whose molecular weight (Tables 1 and 3)

Table 7. *Synthetic polypeptides tested for their immunogenicity*

No. and designation of sample	Antigen added (μg.)/ml. of serum in equivalence zone	Antibody precipitated (μg.)/ml. of serum in equivalence zone
	Immunogenic	
19, p(Tyr,Glu)-pAla--pLys	125	468*
35, p(Tyr,Glu)-pAla--pLys	50	395†
26, p(Tyr,Glu)-pAla--p(Lys,Ala)	50	102†
33, pTyr-pAla--pLys	40	110†
22, p(Tyr,Glu)--pLys	50	451*
23, p(Tyr,Glu)--p(Lys,Ala)	30	150†
106, p(Tyr,Ala)--pLys	25	102†
30, pAla-p(Tyr,Glu)--p(Lys,Ala)	100	247*
120, pAla-p(Tyr,Glu)--p(Lys,Ala)	75	224†
18, pAla-pTyr--p(Lys,Ala)‡	—	—
16, pAla-pTyr--p(Lys,Ala)	75	91†
102, p(Tyr,Glu)	167	2006*
42, p(Tyr,Glu,Ala)	50	403*
44, p(Tyr,Glu,Ala)	50	353†
103, p(Tyr,Glu,Ala)	50	226†
	Non-immunogenic	
20, pAla--pLys	27, pAla-pTyr--pLys	28, pAla-p(Tyr,Glu)--pLys
34, pGlu-pAla--pLys	31, pGlu-pAla-pTyr--pLys	—

* From *E* at 2800 Å of solutions of the precipitates in 0·1 N-sodium hydroxide after deducting the calculated *E* of the antigen. The amount of antigen was obtained from radioactivity data.

† In these cases the antigen was not estimated quantitatively. It was assumed that half the added antigen is present in the immune precipitate.

‡ No precipitation maximum was observed for this system.

Table 8. *Antibody/antigen ratios of precipitates in the equivalence zone*

No. and designation of sample	Mol.wt.	Antibody/antigen in precipitate (w/w)	Antibody/antigen in precipitate (mole/mole)
19, p(Tyr,Glu)-pAla--pLys	70 000*	6·7	2·93
102, p(Tyr,Glu)	12 500†	21·4	1·67
22, p(Tyr,Glu)--pLys	10 200*	23·9	1·52
42, p(Tyr,Glu,Ala)	4 100*	46	1·15
Bovine ribonuclease‡	13 700	—	1·50
Horse serum albumin§	67 000	—	3·50

* From Table 3.
† From Table 1.
‡ From Cinader & Pearce (1956).
§ From Kabat (1961).

and precipitate compositions are known. The molecular weight of the antibody γ-globulin was assumed to be 160 000. It is seen that the weight ratio depends on the molecular weight of the antigen (Boyd, 1956). The molar ratios decrease with the molecular weight, demonstrating that antigens of a smaller molecular weight combine with fewer antibody molecules. The molar ratios of antibody to antigen for bovine pancreatic ribonuclease and horse serum albumin are also included in Table 8, for comparative purposes. There is a remarkable agreement between the molar ratios for synthetic polypeptides and for proteins of similar molecular weights. As the number of combining sites on the synthetic and natural antigens may vary considerably, being probably higher for synthetic polypeptides, it seems that, at least in the range of the molecular weights investigated, the steric factor determines mainly the ratio of antibody to antigen in the equivalence zone.

The first synthetic polypeptide antigen investigated by us (sample 19) contained L-tyrosine, L-glutamic acid, L-lysine and DL-alanine. Among all the polymers subsequently investigated only those containing tyrosine were immunogenic. Substances lacking glutamic acid (e.g. 33, pTyr-pAla--pLys), alanine [e.g. 22, p(Tyr,Glu)--pLys] or lysine [e.g. 102, p(Tyr,Glu)] were shown to be immunogenic. Nevertheless, it must not be concluded that tyrosine is unique in conferring immunogenicity. Previous studies on the increase of the immunogenic capacity of polypeptidyl gelatins as compared with gelatin have demonstrated that attachment of tryptophan or phenylalanine as well as of cysteine or cyclohexylalanine which, in contrast, do not contain aromatic groups, converted gelatin into more powerful antigens. More recently Maurer (1961) and Gill & Doty (1961*a*) have observed that copolymers of glutamic acid and lysine, with a slight residue molar excess of glutamic acid over lysine, are immunogenic.

The serological study of multichain rather than linear polyamino acids offers a most suitable possibility for elucidation of the importance of the locus which the immunogenic sites occupy within the molecule. It may be concluded from the results reported here that the antigenically important area cannot be hidden in the interior of the molecule. It is not necessarily at the end of a peptide chain, since molecules like 30, pAla-p(Tyr,Glu)--p(Lys,Ala), are immunogenic. In this polymer the tyrosine is attached to the backbone of the molecule, but the average distance between the polymeric side chains has been greatly increased in comparison with the non-immunogenic 28, pAla-p(Tyr,Glu)--pLys. The important factor seems to be the accessibility of the particular amino acid (or rigid grouping) during biosynthesis of antibody rather than its presence at the end of a peptide chain. With linear polypeptides the immunogenicity may therefore be attributed to any segment in the chain rather than to the terminal portions only.

The lack of immunogenicity of pAla-p(Tyr, Glu)--pLys, prepared by alanylation of the strongly immunogenic 22, p(Tyr,Glu)--pLys, illustrates the finding that it is possible to convert an antigenic substance into an immunologically inert one by covering densely all of its immunogenic sites. It may also be concluded from this experiment that alanine peptides are not removed *in vivo* to a significant extent from pAla-p(Tyr,Glu)--pLys. Such digestion would, otherwise, release intermediates able to elicit antibodies.

Multichain polymers of different side-chain densities as well as linear polyamino acids may be immunogenic. Thus the overall shape does not seem to be a critical factor in immunogenicity.

Synthetic multichain polyamino acids with molecular weights of about 10 000 are good antigens in rabbits [22, p(Tyr,Glu)--pLys; 23, p(Tyr,Glu)--p(Lys,Ala)]. A linear polymer with a molecular weight around 4000 [42, p(Tyr,Glu, Ala)] is also a good antigen. It is not yet known whether synthetic polypeptides of even lower molecular weights could not be antigenic, provided that they possess the required immunogenic features.

From the studies reported it seems that the investigation of the immunogenic properties of synthetic polypeptides may throw additional light on the minimal requirements for immunogenicity and that such information might be of certain significance in our understanding of the structure and specificity of natural antigens.

SUMMARY

1. A series of 29 linear and multichain copolypeptides, containing all or some of the amino acids tyrosine, glutamic acid, alanine and lysine, were synthesized and characterized.

2. Many of the above-mentioned polymers were tested for their immunogenicity in rabbits. It was found that 15 of the synthetic polypeptides investigated were immunogenic, eliciting between 100 and 2000 μg. of precipitable antibody/ml. of serum.

3. Tyrosine was present in all of the polymers found to be immunogenic.

4. To elicit biosynthesis of antibody the immunogenically important area must readily be accessible, and cannot be hidden in the interior of the molecule.

5. The overall shape of the molecule does not seem to be a critical factor in immunogenicity.

6. The smallest synthetic polypeptide antigen among the substances investigated was a linear polymer of an average molecular weight around 4000.

7. From the molar ratios of antibody to antigen in the equivalence zone it may be concluded that the number of antigenic specific sites on the molecule decreases with the molecular weight of the synthetic antigen.

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